

Phylogeography of the genus *Erebia* (Lepidoptera, Nymphalidae) in Bulgaria, inferred by mitochondrial DNA

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Abstract Phylogeographical analysis was conducted on butterflies of the genus *Erebia* in Bulgaria, which occupies a major position on the Balkan Peninsula. We studied the haplotypes of base sequences of 942 bp in total using *ND5* (432 bp) and *COI* (510 bp) which are often used for analysis of intraspecific molecular variation. Samples are 65 individuals of 10 *Erebia* species from northern Bulgaria (Western and Central Balkan Mts) and southern Bulgaria (Rodopi, Pirin and Rila Mts), and also 11 individuals of 10 species from central Europe mainly Switzerland and central Asia.

A geographical distance-based isolation mechanism may be functioning for *E. aethiops*, *E. euryale*, and *E. medusa*, which are widely distributed in Europe, with just a one-base substitution in the sequence between the north and south populations of *E. aethiops*, and the other north-south species being in an equivalent situation. On the other hand extremely large genetic differences between the north and south Bulgarian populations of *E. ligea* and *E. oeme* have been discovered. This is probably a result of reproductive isolation between the north and south populations prior to the last glacial period which remains till now. Especially with *E. ligea*, it may be that two lineages, the European and Asian, are facing each other in the central Balkan Peninsula. In addition, in the *E. alberganus* and *E. pandrose* populations, reproductive isolation between the Balkan populations and central European populations seems to have continued since the age prior to the last glacial stage, since the genetic differences between them are very large. This suggests that some northern butterflies like these two *Erebia* species, now distributed very sporadically, might not have expanded their distribution and neither mixed each other in the last glacial period even on the continent.

Key words Biogeography, Quaternary period, Balkan Peninsula, genus *Erebia*, mitochondrial DNA, *ND5*, *COI*.

Introduction

The Balkan Peninsula have been known as a hotspot for European biodiversity (Griffiths *et al.*, 2004) since, together with the Iberian and Italian Peninsulas, it functioned as a refugium for southern (Mediterranean) organisms during the ice age (Hewitt, 2000). The Balkan Peninsula generally indicates an area south of the Danube river. On the western side of the Peninsula, the Dinaric Alps stretch from northwest to southeast, and are among the five largest mountain ranges in Europe, with Prokletije (2,692 m) as the highest peak. At the southernmost end, the mountains are divided to two mountain ranges, the Balkan Mountains (highest peak Betov, 2,376 m) running from west to east, and the Pindos Mountains (highest peak Katafidi, 2,393 m) running from north to south. There is a high level of biodiversity compared to their rather narrow regional areas, such as the butterfly fauna with 214 species in Bulgaria (Abadjiev, 2001), and 232 in Greece (Pamperis, 1997).

In studies on northern (non-Mediterranean) organisms, however, some cases have been reported of organisms having small refugia in the far northern region other than the Mediterranean, corresponding to the strength of cold tolerance of the organisms (*e. g.* Steward and Lister, 2001; Schmitt *et al.* 2006). Although there have been few studies

on northern organisms, reported cases show more varied patterns in terms of such factors as the locations of refugia during the warm periods and dispersal routes during the ice age than is the case with southern organisms (Hewitt, 1996; Schmitt, 2009).

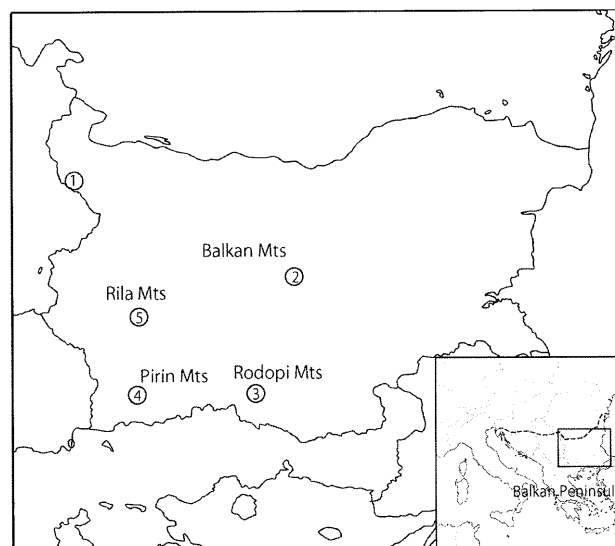


Fig. 1. Balkan Peninsula. Map showing the approximate locations of the sampling sites.

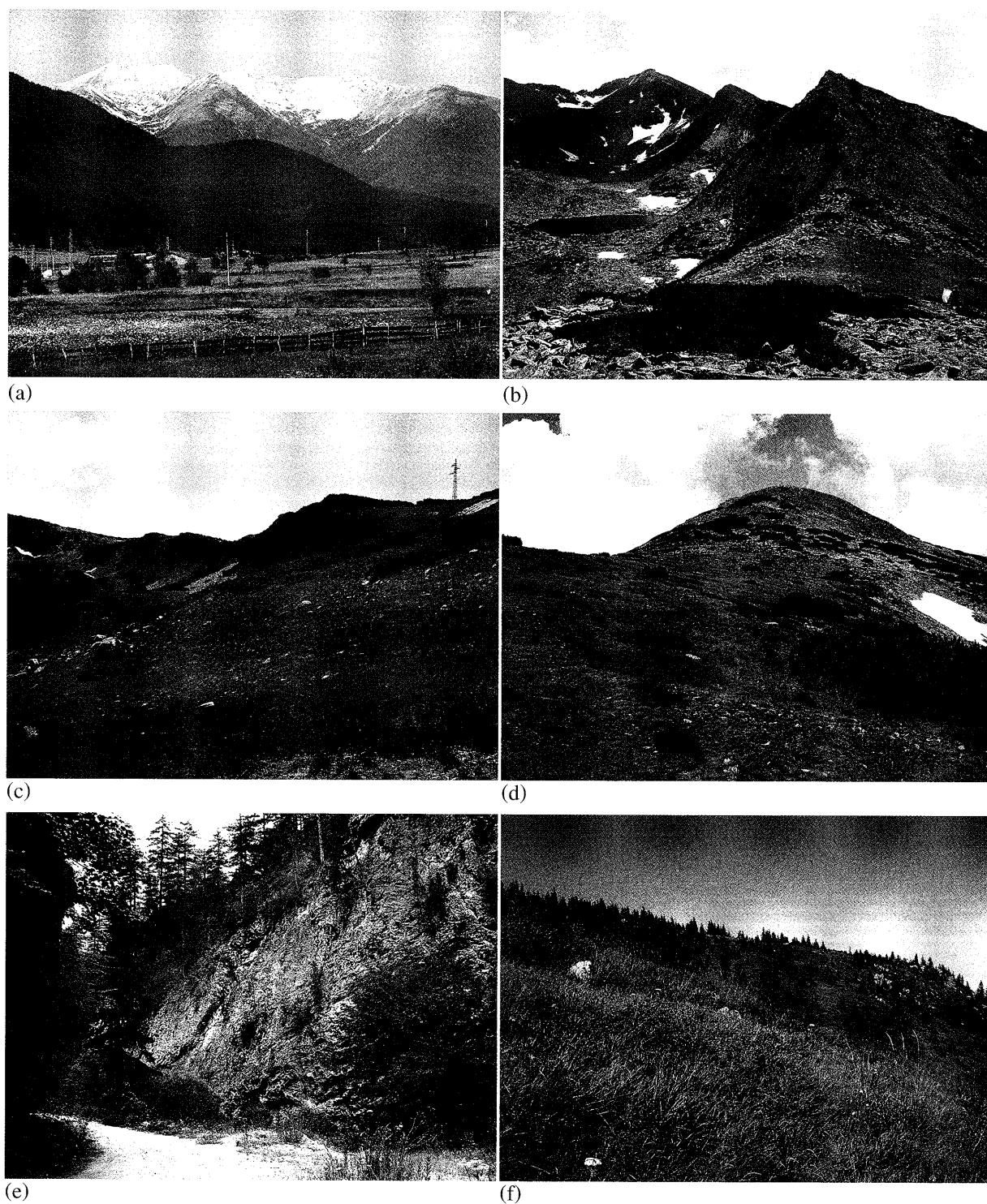


Fig. 2. Habitats of the genus *Erebia*

(a) Rila Mts, distant view, (b) Rila Mts, Mt. Borovets (h=2,550 m). *E. pandrose* on slope areas with short grasses and rocky outcrops. (c) Rila Mts, Grqncchar Chalet-Dzhanka Pass (h=2,500 m). *E. orientalis* and *E. oeme* on grassy slopes near the timberline. (d) Pirin Mts, Bezbug Chalet (h=2,650 m). Many *E. rhodopensis* on the boundary areas of rocky outcrops and short grass areas. (e) Rodopi Mts, Devin, Trigrad (h=1,600 m). *E. euryale* and *E. ligea*. (f) Western Balkan, Chiproviska Planina (h=1,350 m). *E. orientalis*, *E. alberganus*, *E. ottomana* on tall grassy and flowery slopes with sporadic low coniferous trees.



Fig. 3. *Erebia* butterflies in the field of Bulgaria

(a) *Erebia ottomana* (Pirin Mts, Gotse Delchev-Sandanski, 21 July 2009)

(b) *Erebia orientalis* (Western Balkan, Chiproviska Planina, 14 July 2009)

(c) *Erebia euryale* (Pirin Mts, Gotse Delchev-Sandanski, 21 July 2009)

(d) *Erebia ligea* (Rodopi Mts, Devin, Trigrad, 18 July 2009)

(e) *Erebia alberganus* (Western Balkan, Godech, Petrohan Pass, 13 July 2009)

(f) *Erebia rhodopensis* (Rila Mts, Borovets, Moussala, 22 July 2009)

Schmitt *et al.* (2006) studied the genetic structure of populations of the alpine butterfly *E. epiphron* over its range of distribution. The strongly hierarchical genetic structure among populations geographically distant from each other led them to surmise that these populations were already reproductively isolated in the last ice age rather than that the present distribution of local populations had been formed in the short postglacial period. Due to the extreme dry climate of the ice age, some alpine butterflies which preferred humid environments were incapable of wide distribution even in the cooler age, and may have been sporadically distributed in regions in which humidity was highly preserved microclimatically. The same patterns are observed in alpine plants (Schonswetter *et al.*, 2003a, 2003b).

Haubrich and Schmitt (2007) examined the genetic structure of the alpine-endemic butterflies *E. sudetica* and *E. melampus*. They detected strong differentiation into three lineages with the genetic distances between the two *E. melampus* groups being larger than between each of the two *E. melampus* groups and *E. sudetica*. These results give evidence for the existence of a species complex within the *E. melampus* / *sudetica* group and indicate a discontinuous distribution within this group during at least the last ice age. One of them, *E. sudetica*, is found in the northern Alps and most probably had its refugium north of the glaciated Alps. The western *E. melampus* group might have had a refugium at the margin of the southwestern Alps, and the eastern group in the lower altitudes of the southwestern and/or eastern Alps.

Erebia species inhabiting the coniferous forest zone are considered not to have inhabited environments lacking coniferous forests even in the ice age. Schmitt and Haubrich (2008) examined the genetic structure of populations of forest-dwelling *E. euryale* in four different European mountain systems (Pyrenees, Alps, Carpathians, and Rila). They found four different groups (Pyrenees, western Alps, eastern Alps, and southeastern Europe) well supported by many analysis methods. The genetic diversity of the populations was highest in the southeastern European group and decreased stepwise westwards. They assume that the most important refugia for the coniferous forest biome in Europe was located in southeastern Europe, and that a fragment of this habitat type survived along the foothills of the Alps and Pyrenees.

Allozyme analysis of *E. medusa*, more widely distributed in the lower altitude areas than *E. euryale* over large regions of Europe, revealed a significant population differentiation (Schmitt and Seitz, 2001). Phylogenetic analysis showed a division into four major lineages, the eastern group including the Czech Republic, Slovakia and Hungary, the

central group France and Germany, the lineages being delimited from western Hungary, and from northern Italy. The genetic difference between the populations is small, probably arising from disjunct distribution during the last ice age. Genetic lineages evolving during glacial isolation frequently come into contact as the result of post glacial range expansions. Hybridization often occurs along these contact zones. Hybrid populations between the eastern and the western genetic lineage of *E. medusa* were detected in the Hercynian mountain area along the Czech-German border (Schmitt and Muller, 2007). Northern butterflies that had less strong cold tolerance were forced into vicariance during the ice age before expanding their distribution during the postglacial period.

Evidence of four different Quaternary glacial stages has been found in the north-west Iberian Peninsula. The phylogeographic history of Iberian *E. triaria* was analysed (Vila *et al.*, 2005). One of the NW Iberian populations, a subspecies endemic to the Xistral mountain range, showed a high level of genetic divergence from other populations. Isolation after an ancient glacial stage, followed by allopatric differentiation, may account for such high differentiation. The genetic pattern shown by the rest of population samples consisted of two lineages, likely reflecting that the ancestors of these populations sought refuge in at least two different areas during a subsequent glacial age. If an analysis is conducted with many more sample points, multiple events may be inferred, including range expansion and/or fragmentation, as in the case of *E. niphonica* in Japan (Nakatani *et al.*, 2007).

In this paper we discuss the phylogeographic history of the genus *Erebia* in Bulgaria, the core of the Balkan Peninsula, by comparing the alpine butterflies on the Japanese islands.

Materials and Methods

The authors collected 65 individuals of 10 species from the northern mountain systems (Western and Central Balkan Mts) and southern mountain systems (Rodopi, Rila and Pirin Mts) in Bulgaria, and 11 individuals of 10 species from central Europe, mainly Switzerland, and central Asia. The bodies of the sample specimens without wings were preserved in 99% ethanol and kept at 4°C until DNA was extracted.

For genetic markers, we used a part of the NADH dehydrogenase subunit 5 (*ND5*) and cytochrome oxidase subunit I (*COI*) genes in mitochondrial DNA. DNA was extracted from the thoracic flight muscles or legs by DNeasy Tissue Extraction Kit (Qiagen, Hilden, Germany). PCR was conducted in a 10 µl reaction system by adding

Table 1. Haplotype list.

Detected locality in Bulgaria; WB: West Balkan. CB: Central Balkan, RO: Rodopi Mts, PI: Pirin Mts, RI: Rila Mts

Haplotype		Sampled locality	Accession No.		n	Detected locality in Bulgaria				
			ND5	COI		WB	CB	RO	PI	RI
<i>E. aethiops</i>										
AE000	Swiss: Goppenstein, Wallis	AB674329	AB674366	1						
AE010	Bulgaria: Chiproviska Planina, West Balkan	AB674330	AB674367	2	○					
AE011	Bulgaria: Rozhen Pass near Smolyan, Rodopi	AB674331	AB674368	3				○		
<i>E. alberganus</i>										
AL000	Swiss: Zermatt, Wallis	AB674332	AB674369	1						
AL010	Bulgaria: Chiproviska Planina, West Balkan	AB674333	AB674370	2	○	○				
<i>E. euryale</i>										
EU000	Swiss: Gross Scheidegg, Bern	AB674334	AB674371	1						
EU010	Bulgaria: Orelyak, Pirin	AB674335	AB674372	5	○			○	○	
EU011	Bulgaria: Chiproviska Planina, West Balkan	AB674336	AB674373	1	○					
EU012	Bulgaria: Betov, Central Balkan	AB674337	AB674374	1		○				
EU013	Bulgaria: Pomporovo Resort, Rodopi	AB674338	AB674375	1				○		
EU014	Bulgaria: Trigrad Gorge, Rodopi	AB674339	AB674376	2				○		
<i>E. ligea</i>										
LI000	Swiss: Goppenstein, Wallis	AB674340	AB674377	1						
LI001	Swiss: Maienfeld, Graubunden	AB674341	AB674378	1						
LI100	Mongolia: Bayanchandman, Tov	AB674342	AB674379	1						
LI010	Bulgaria: Chiproviska Planina, West Balkan	AB674343	AB674380	2	○					
LI011	Bulgaria: Chiproviska Planina, West Balkan	AB674344	AB674381	1	○					
LI012	Bulgaria: Chiproviska Planina, West Balkan	AB674345	AB674382	3	○					
LI013	Bulgaria: Trigrad Osmanov, Rodopi	AB674346	AB674383	5				○		
<i>E. medusa</i>										
ME000	Germany: Baden-Wurttemberg	AB674347	AB674384	1						
ME100	Mongolia: Terelji, Tov	AB674348	AB674385	1						
ME010	Bulgaria: Central Balkan, Pirin(Mt. Bezbog)	AB674349	AB674386	3			○		○	
ME011	Bulgaria: Central Balkan	AB674350	AB674387	1			○			
<i>E. oeme</i>										
OE000	Swiss: Spillmatten, Wallis	AB674351	AB674388	1						
OE010	Bulgaria: Berkoviska Planina, West Balkan	AB674352	AB674389	2	○					
OE011	Bulgaria: Berkoviska Planina, West Balkan	AB674353	AB674390	1	○					
OE012	Bulgaria: Pomporovo Resort, Rodopi	AB674354	AB674391	3				○	○	
OE013	Bulgaria: Pomporovo Resort, Rodopi	AB674355	AB674392	1				○		
<i>E. ottomana</i>										
OT000	Italy: Monte Baldo	AB674356	AB674393	2						
OT010	Montenegro: Durmitor	AB674357	AB674394	4	○	○			○	
OT011	Bulgaria: Orelyak, Pirin	AB674358	AB674395	2						
OT012	Bulgaria: Granchar-Chalet Pass, Rila	AB674359	AB674396	2					○	
OT013	Bulgaria: Pomporovo Resort, Rodopi	AB674360	AB674397	3				○		
OT014	Bulgaria: Pomporovo Resort, Rodopi	AB674361	AB674398	1				○		
<i>E. pandrose</i>										
PA000	Italy: Piemonte	AB674362	AB674399	1						
PA010	Bulgaria: , Borovets, Rila	AB674363	AB674400	4					○	
<i>E. rhodopensis</i>										
RH010	Bulgaria: Borovets, Rila	AB674364	AB674401	8		○		○	○	
RH011	Bulgaria: Granchar-Chalet Pass, Rila	AB674365	AB674402	1					○	

Table 2. Genetic distances as shown by the numbers of base substitutions. (a) Alps and Balkan Peninsula (b) Honshu (Japanese islands) and Continent.

(a)

Species	Alps-Balkan
<i>Erebia pamdrose</i>	6
<i>Erebia oeme</i>	5
<i>Erebia alberganus</i>	4
<i>Erebia ligea</i>	2
<i>Erebia ottomana</i>	2
<i>Erebia medusa</i>	2
<i>Erebia euryale</i>	2
<i>Erebia aethiops</i>	1

(b)

Species	Continent-Honshu	Locality in Continent
<i>Carterocephalus palaemon</i>	7	Russia: Khabarovsk
<i>Anthocharis cardamines</i>	6	Russia: Komsomol'sk na Amure
<i>Colias palaeno</i>	5	Russia: Yakutia, Ust'-Nera

DNA (ca 40 ng) with reaction constitution as recommended by Applied Biosystems. Thermal cycling profile was one step at 95°C for 10 min; 25 cycles at 95°C for 30 sec, 45°C for 30 sec and 72°C for 1 min 30 sec; one extension step at 72°C for 8 min 30 sec. PCR products were processed after Exo-SAP treatment. The nucleotide sequences of the amplified DNA fragments were determined by the direct sequencing method with Big Dye Terminator v1.1 Cycle Sequence Kit. As primers for PCR and direct sequencing, we used V1 (5'-CCT GTT TCT GCT TTA GTT CA-3'), C2 (5'-ATC YTT WGA ATA AAA YCC AGC-3') (Yagi *et al.*, 1999) for *ND5*, and mtD6 (C1-J-1718; 5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3'), Nancy (C1-N-2191; 5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3') (Simon *et al.*, 1994), or LCO (5'-GGT CAA CAA ATC ATA AAG ATA TTG G -3'), HCO (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA -3') (Folmer *et al.*, 1994) for *COI*. Sequences were edited and aligned using SeqScape V. 2.11 (ABI, Weiterstadt, Germany). We conducted fragment comparison of 432 bp for *ND5*, 510 bp for *COI*, and 942 bp in total. A haplotype analysis for the gene dataset and construction of a haplotype network were conducted using TCS v. 1.21 (Clement *et al.*, 2000).

Results

The *ND5* and *COI* haplotypes detected are shown in Table 1, and haplotype network trees obtained from the sequences of each species are shown in Fig. 4. *E. ottomana* was the only species having a variable haplotype in each mountain range. Other species were roughly divided into two lineages, the northern (Balkan Mountains) and the southern (Pirin, Rila, Rodopi). Only *E. rhodopensis* exhibited a very few

haplotypes despite the considerable sample numbers analyzed, with no significant differences in haplotype diversity among the other species.

The following three patterns were noticed by comparing the genetic distance among populations within Bulgaria, with the genetic distance between the populations in Bulgaria and those in the central Europe.

- (1) Genetic distance is generally proportional to geographical distance
- (2) Genetic distance is markedly large, without any clear correlation with geographical distance
- (3) Genetic distance is very large between the Bulgarian and central European population

Discussion

1 Three patterns observed in genetic distance between Bulgarian and central European populations

- (1) Pattern of genetic distance generally proportional to geographic distance

This is a pattern in which a distance-based isolation mechanism (island model) is presumed to be functioning, with genetic exchange observed among neighboring populations but not among distant populations. In this group, *Erebia aethiops*, *E. euryale* and *E. medusa* are included. These species are all widely distributed and inhabit grasslands in coniferous forest zones. The elevation of habitats confirmed by the present authors' field survey were 950–1,000 m (West Balkan) and 1,460 m (Rodopi) for *E. aethiops* and 950–1,000 m (West Balkan) for *E.*

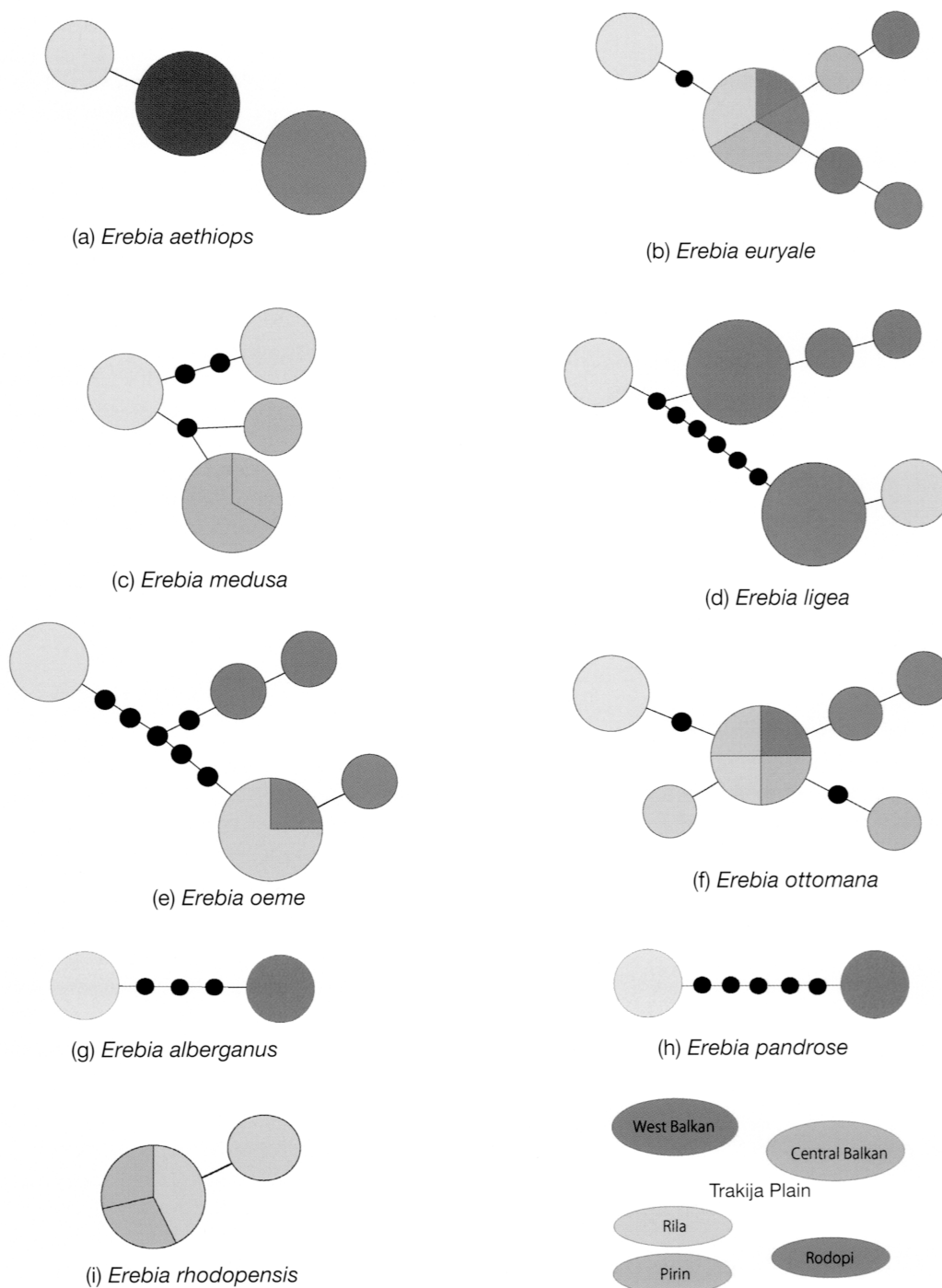


Fig. 4. Haplotype networks of mitochondrial *ND5* and *COI* sequences.

The size of circles indicates sample size of each haplotype (small circle, $n \leq 2$, large circle, $n > 2$). One node indicates a single base substitution and a small filled circle indicates a missing haplotype.

euryale. *E. medusa* was only found in alpine meadows at 1,680–2,000 m (Central Balkan) and 2,380–2,400 m (Pirin) in July, although it is observed in places at around 500 m in its early (May) appearance in Bulgaria (Abadjiev, 1993).

This pattern is easily understood based on the biological characteristics of these species. The distribution ranges of these species would be expanded together with the expansion of the forest zone in the postglacial period, while they were fragmented in the ice age.

- (2) Pattern in which genetic distance is markedly large, with no correlation with geographical distance

This pattern would be caused by two populations forming a hybrid zone or facing each other in a close distance resulting from the subsequent range expansions after the reproductive isolation in multiple refugia. Some unknown phylogeographical factors were suggested. *E. oeme* and *E. ligea* are included.

E. ligea inhabits similar environments to those of *E. aethiops* and *E. euryale* of the above mentioned group. While it often is observed sympatrically with these species, there is an extremely large genetic distance between populations in northern and southern Bulgaria. In addition the Balkan Mountains haplotype is genetically very closely related to Switzerland populations, and the south Bulgarian populations to geographically distant central Asian (Mongolian) populations. *E. ligea* is widely distributed from Europe along the mid-latitude regions of the Eurasian continent to the Russian Far East and the Japanese islands. The implication is that a ancestral panmictic population at an early glacial period divided into two separate populations of the eastern lineage and western lineage as a result of isolation in more than two refugia during the following warm interglacial period. It is presumed that these two lineages expanded their distribution areas followed by confronting each other in the central Balkan Peninsula. As regards populations in the Japanese islands, about 15 base substitutions are recognized compared with the Mongolian population. More samples from various points covering the whole distribution ranges are necessary to clarify its phylogeographic history.

E. oeme, endemic to the European regions, is limited to grasslands in coniferous forests in the central European Alps. In Bulgaria many populations are also widely distributed in the grasslands above the timberline. The distribution range is spread from the Pyrenees in the west to the Carpathians and Balkan Peninsula in the east, and many subspecies and forms are recognized. Although the populations in the Balkan Peninsula together with those from eastern Austria are classified as the same subspecies *spodia*, having a larger wing span and well developed

orange-red markings, there is a great genetic distance between the populations in northern and southern Bulgaria, with no correlation to the geographic distances and similarity of morphological characters. Two lineages in northern and southern Bulgaria might have been isolated in separate refugia prior to the last ice age.

- (3) Pattern with larger genetic distance from central European populations

This group includes *E. pandrose* and *E. alberganus*. These species have large genetic distances from the central European populations, but a comparison was not possible between the north and south Bulgarian populations in this study. The distribution ranges of each species might have been fragmented prior to the last ice age, and apparently there might have been no genetic exchanges in or after the following cool period up to the present time. Both species inhabit grasslands near the timberline, and *E. alberganus* also inhabits grasslands in coniferous forests at a lower elevation, but the distribution ranges of these species are sporadic. Species having a similar distribution pattern have presumably remained genetically isolated from prior to the last ice age until today.

2 Species endemic to the Balkan Peninsula

Species endemic to the Balkan Peninsula include *E. rhodopensis*, *E. orientalis* and *E. melas*, and among them only *E. rhodopensis* and *E. orientalis* were analyzed in this study. The Balkan Peninsula serves as the main distribution area for *E. ottomana*, which was also treated as belonging to this group.

Closely related to *E. aetheopella* in central Europe, *E. rhodopensis* is mainly distributed in the shrub zone near the timberline and also inhabits in a stony or rocky grassland above 2,650 m elevation in Bezbug Chalet (Pirin Mts) and Grqnchar Chalet (Rila Mts). Genetic variation was not detected in this study.

E. ottomana belongs to the *tyndarus* group which is well diversified in Europe. Its main distribution area is the Balkan Peninsula and is also locally distributed in southeastern France and northern Italy. The widely distributed haplotype was found from the populations of Balkan Mountains in the north and Rila Mts in the south, and also detected from the population in Montenegro. The star-shaped network tree (Fig. 4) contains this widely distributed haplotype at the center and a single-base substitution haplotype distributed in the Rodopi Mts and two-base substitution haplotype distributed in the Pirin Mts and central Europe (Monte Baldo, Italy) are derived from the main one. Within the *tyndarus* group, this species belongs to a relatively ancient lineage with *E. iranica* and *E. graucasica* distributed in the Near East, and seems to

have expanded its distribution from the Balkan Peninsula to central Europe (northern Italy and southeastern France).

3 History of genetic differentiation

Here we dare to discuss the relatedness between the geographical age when populations of some *Erebia* species in the Balkan Peninsula were reproductively isolated from the central European populations and that when the Japanese alpine butterflies were genetically isolated from populations on the continent. The Balkan Peninsula has been a part of the European continent all through the Quaternary period (Hewitt, 2000). Honshu, the main island of the Japanese islands, however, has been separated from Hokkaido to the north by the Tsugaru Strait and from the Korean Peninsula to the southwest by the Tsushima Strait. These two straits are deep. Honshu separated from Hokkaido and the Korean Peninsula and became an island during the interglacial age after Riss glaciation about 150,000 years ago. It is believed that Honshu has not been connected with the continent since then (Oshima, 2000). In other words, alpine organisms which now show isolated distribution in the alpine belt of Honshu had expanded their distribution from the continent prior to about 150,000 years ago, and they are the species which were affected by the climatic changes since then during the interglacial period and the last glacial period. At least two periods, when glaciers developed, existed in the high altitude areas of central Honshu in the Japanese islands, and even now there are mountainous permafrosts in some limited areas in the high mountainous areas in the Japanese islands (Iwata, 2003).

The same base sequences of *ND5* and *COI* regions used in this study were also used in the previous studies on the genetic differences of the Japanese alpine butterflies from the populations on the continent (Nakatani *et al.*, 2005, 2007a, 2007b), and it allows us to compare the geographical age when the Balkan populations were reproductively isolated with that when the Japanese alpine butterflies were isolated by using the base substitution number.

The base substitution numbers detected are summarized in Table 2. The base substitution numbers between the central European and Bulgarian populations are 4–6 for the sporadically distributed species: *Erebia pandrose* and *alberganus* (Table 2a). For the alpine butterflies in Honshu, *Anthocharis cardamines*, *Colias palaeno*, and *Caterocephallus palaemon*, the base substitution numbers between continental and Honshu populations are 5–7 (Table 2b). These data suggest that the Bulgarian populations of the sporadically distributed *Erebia* species group were reproductively isolated from the central European populations during the cooler period prior to the last glacial age. This implies that not all northern butterflies were able

to expand their distribution even in the cooler periods on the continent. It also suggests that similar climatic environments would have caused such a disjunct distribution for northern butterfly populations during almost the same period both in Europe and in eastern Asia.

On the other hand, the base substitution numbers between the central European and Bulgarian populations are 1–2 for the widely distributed species *E. aethiops*, *E. euryale* and *E. medusa*. This indicates that genetic exchanges between the central European and Bulgarian populations of these species had continued up until the last glacial period. In addition, genetic exchanges continued until very recently between the northern and southern populations in Bulgaria for each of these three widely distributed species and in addition for *E. ottomana* originating in the Balkan Peninsula and for *E. rhodopensis* endemic to the Balkan Peninsula.

The vast phylogenetic divergence of *E. ligea* in the Balkan Peninsula is extremely interesting. It is important to clarify the genetic structure of this species by analyzing more samples from various places covering the entire main distributional areas in a future study.

Acknowledgments

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摘 要

ブルガリアにおけるベニヒカゲ属蝶類の系統地理
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バルカン半島は、イベリア半島、イタリア半島と共に氷河時代には、南方系生物にとってレフュジアとして機能していたため、ヨーロッパ産生物多様性のホットスポットとして知られる。バルカン半島とは一般にドナウ川より南を指す。西側にはDinaric Alpsが北西から南東方向に連なり、最高峰はProkletije (2,692 m) でヨーロッパで五指に入る大山

脈である。その南端では東西に連なる Balkan Mts（最高峰は Betov, 2,376 m）と、南北に連なる Pindos Mts（最高峰は Katafidi, 2,393 m）に分かれる。蝶類はブルガリア 214 種、ギリシア 232 種に達し、地域の面積からすると蝶類の種数でも多様性が高いことが伺われる。

サンプルとして、ブルガリアの北部 (West and Central Balkan Mts) および南部 (Rodopi, Pirin, Rila Mts) で採集した *Erebia* 属 10 種 65 個体、およびスイス、イタリアなど中欧、および中央アジア産の 10 種 11 個体を用いた。ミトコンドリアの遺伝子領域としては、種内変異の解析によく使われる *ND5* の一部 (432 bp) と *COI* 領域の一部 (510 bp)、合計 942 bp を使用し、得られた塩基配列を比較してハプロタイプを決定した。

ブルガリア国内産と中欧産個体群間の遺伝距離にみられるパターン

ブルガリア国内におけるハプロタイプの分化と中欧産との遺伝距離を比較すると、3つのパターンに分類することができる。

(1) 遺伝距離がほぼ地理的距離に比例しているパターン

近隣個体群間では遺伝的交流が認められるが、遠距離の個体群間では認められない、いわゆる距離による隔離機構（島モデル）が働いていると想定されるパターンである。このグループに属する種としては、*E. aethiops*, *E. euryale*, *E. medusa* がある。いずれも針葉樹林帯の草原に生息する広域分布種で、その生態的特性からも推定されるパターンであって、氷河期に分布が分断されたものが、温暖期における森林帯の拡大に伴って分布を広げたと推定される。

(2) 個体群間の地理的距離とは相関のない遺伝距離が認められるパターン

過去に複数のレフュジアに生殖隔離されることで固定された複数の系統が、その後に分布を拡大して混生地を生じたり、あるいは地理的に至近距離で対峙している分布型であり、何らかの系統地理的要因のあることが示唆される。*E. oeme*, *E. ligea* が属する。

E. ligea は前のグループに属する *E. aethiops*, *E. euryale* と類似の環境に生息し、しばしばこれらの種と同所的に見られる場合が多いにもかかわらず、ブルガリア北部のバルカン山脈産と南部の山系産の個体群間の遺伝距離はきわめて大きい。さらにバルカン山脈産のハプロタイプはスイス産個体群と、またブルガリア南部山系産個体群は地理的に遠く離れた中央アジア（モンゴル）産の個体群と遺伝的に極めて近縁であることが判明した。*E. ligea* はヨーロッパからユーラシア大陸の中緯度地域に沿ってロシア極東、日本列島にまで広域分布する種であるが、東西2系統に分断された個体群が、その後に分布を拡大しバルカン半島の中部で両系統が対峙していると想定される。今後大陸各地においてサンプル地点を増やすことで系統地理的な全貌を解明する必要がある。

E. oeme はヨーロッパ地域特産で、中欧のアルプスでは針葉樹林内の草原に生息し分布は局限されるのに対して、ブルガリアでは森林限界より上の草原にも広く分布し個体数も多い。西はピレネー山脈から東はカルパチア山脈・バルカン半島に分布し、いくつかの亜種またはフォームに区分される。バルカン半島産は東オーストリア産と共に大型で、斑紋がよく発達する特徴を持ち亜種 *spodia* とされるが、北部ブルガリア産と南部ブルガリア産個体群の間の遺伝距離は大きく、遺伝距離と形態的特徴の類似性には相関性がない。両者の個体群は、その遺伝距離の大きさからみると最終氷河期以前から別々のレフュジアに隔離分布していたことが示唆される。

(3) 中欧産個体群との遺伝的距離が大きいパターン

中欧産個体群との遺伝距離が大きいグループである。最終氷期よりも前に分布が分断され、その後の寒冷期にも遺伝的交流がないまま現在に至っていると想定される。*E. pandrose*, *E. alberganus* が属する。*E. pandrose* は森林限界付近の草原に生息するが、*E. alberganus* はさらに低標高の針葉樹林内の草原にも生息する。スイスでは高山草原でも草丈の高い草原にのみ見られ、食草が限定されているのかもしれない。このような離散的分布域をもつ北方系蝶類の一部の種は、最終氷期よりも前に分布が分断され、その後の寒冷期にも分布を拡大することができなかったと推定される。

バルカン半島固有種

バルカン半島には *E. rhodopensis*, *E. orientalis*, *E. melas* の固有種が知られているが、これらの内 *E. rhodopensis*, *E. orientalis* について解析できた。バルカン半島が主たる分布地の *E. ottomana* もこのグループに属するものとして扱う。

E. rhodopensis は中欧に分布する *E. aetheopella* と近縁であり、森林限界付近の灌木帯に分布の中心があり、Bezbug Chalet (Pirin Mts) や Grqncchar Chalet (Rila Mts) では標高 2650 m 以上の岩礫帯でもみられた。調査した範囲では遺伝的変異は認められなかった。

E. ottomana は中欧で多様な種分化を遂げた *tyndarus* グループに属し、フランス南東部とイタリア北部にも分布するが、バルカン半島が主たる分布域である。広域分布型のハプロタイプが北部バルカン山脈と南部 Rila 山系から見出され、同じ型はモンテネグロ産個体群からも検出された。ネットワーク樹に示すように、この広域分布型ハプロタイプを中心にして、1塩基置換のハプロタイプが Rodopi Mts に、2塩基置換のタイプが Pirin Mts と中欧（イタリア、Monte Baldo）に分布するスター型の構造を示す。本種は *tyndarus* グループの中では近東に分布する *E. iranica*, *E. graucasica* と共に古い系統に属する種であって、バルカン半島から中欧（イタリア北部、フランス南東部）へ進出したものであろう。

E. orientalis は Rila Mts のみから得られたため、ブルガリア南北の個体群間の比較はできなかった。

遺伝的分化の歴史

日本産高山蝶の各種については、筆者らによる大陸産の同種と遺伝的差異を比較検討した研究、および日本列島内でのハプロタイプを解析した研究がある。本州は最終氷期には大陸や北海道と陸続きにならなかったため、本州に遺存分布する高山蝶は最終氷期より前の寒冷期に大陸から渡来したものと考えられる。したがって本州の例と比較することで、バルカン半島での生殖隔離が始まった年代を推定することができる。

分析した遺伝領域 *ND5* と *COI* の塩基置換数をまとめると Table 2 のようになる。中欧とブルガリア産個体群間の塩基置換数は、分布が離散的な *E. pandrose* と *E. alberganus* は 4～6 である (Table 2a)。一方本州産高山蝶のクモツマキチョウ、ミヤマモンキチョウ、タカネキマダラセセリは、大陸産と本州産個体群の塩基置換数が 5～7 である (Table 2b)。これらのデータから、離散分布型の *Erebia* 属蝶類が中欧とブルガリア間で生殖隔離した時期は、最終氷期より前の寒冷期であると示唆される。すなわちヨーロッパとアジア東部において、ほぼ同時期に北方系蝶類の個体群が隔離分布するような気候環境であったことが示唆される。また

これらの種の中欧とバルカン半島の個体群が、最終氷期を通じて遺伝的交流がなかったことは、寒冷期とはいえ北方系蝶類のすべてが分布域を拡大できたわけではないことを示唆している。

同じく Table 2a によると、中欧とブルガリア産個体群間の塩基置換数は、広域分布型の *Erebia aethiops*, *E. euryale*, *E. medusa* では 1～2 であり、最終氷期以降も遺伝的交流があったことが示唆される。またブルガリアの南北個体群間では、広域分布型の *Erebia aethiops*, *E. euryale*, *E. medusa*, およびバルカン半島が発祥の地と推定される *E. ottomana*, バルカン半島固有種の *E. rhodopensis* においては、*E. aethiops* 以外は広域分布型のハプロタイプが南北の山域に共通して生息しており、ごく最近まで遺伝的交流のあったことが示唆される。

バルカン半島で系統分岐の推定された *E. ligea* は系統地理的に極めて興味深い事象であって、今後さらにサンプリング地点を増やして、主要な分布域をカバーする集団の遺伝的構造を明らかにする必要がある。

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